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Progress in the Isolation and Characterization of a Host Hemolymph Ovipositional Kairomone for the Endoparasitoid *Microplitis croceipes*

Robert R. Heath, Stephen M. Ferkovich, Patrick D. Greany, Fred J. Eller, Barbara D. Dueben, and Robert L. Tilden

Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, USDA, Gainesville, Florida

The purpose of this study was to 1) define further the bioassay parameters of an ovipositional stimulating kairomone (OSK) for *Microplitis croceipes* found in the hemolymph of the corn earworm, *Heliothis zea*; 2) purify and isolate the OSK; and 3) determine the viability of the eggs oviposited into an artificial ovipositional substrate (AOS). Phenylthiourea (PTU), added to hemolymph to prevent melanization, and host feces which contains a host-seeking stimulant (Jones et al.: *Sciences* 173:842, 1971) [1], were eliminated as possible factors influencing egg laying in the bioassay. Extraction of hemolymph with ether, hexane, and to a lesser degree with methylene chloride removed lipids without loss of OSK activity. In contrast, extraction with polar solvents such as methanol tetrahydrofuran, and acetonitrile resulted in a loss of OSK activity. After ether extraction, the sample could be concentrated by rotoevaporation (90°C) or lyophilization without loss of OSK activity. Fractionation of the sample by gel permeation chromatography indicated a molecular weight of between 100–300 daltons. The OSK was extracted to a specific activity higher than crude hemolymph on two disposable solid-phase adsorbants, a normal-phase diol, and a reverse-phase phenyl material. Subsequent fractionation of hemolymph on a phenyl adsorbent column by HPLC indicated that the OSK contained at least two components. Ovipositional activity was obtained only when two fractions with different retention volumes were combined. Preparation of the AOS's from agar plus Goodwin's tissue culture medium maintained viability of the oviposited eggs. Thirty-seven percent of the eggs that were removed from the AOS's and held in culture media eventually hatched.

Key words: hemolymph, *Heliothis zea*, artificial ovipositional substrate, parasitoid, egg collection, viability, HPLC, solid-phase adsorbents

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Address reprint requests to R.R. Heath, P.O. Box 14565, Gainesville, FL 32604.

INTRODUCTION

The endoparasitoid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae) is an important parasitoid of the bollworm, *Heliothis zea* (Boddie) , and the tobacco budworm, *H. virescens* (F.) in the southern region of the United States [2,3]. High rates of parasitism of these two economically important pests have been reported in field crops, especially cotton [4–6]. The parasitoid also has been reported to have a high tolerance to commonly used insecticides [7]. Considerable attention has been directed to its population dynamics, behavior, physiology, and potential for artificial culture because of the parasitoid's potential use as a biological control agent for *Heliothis* spp. in pest management programs [8–21]. Use of the parasitoid in field release programs depends on the development of techniques for augmenting and manipulating the performance of the parasitoid. Efforts toward development of an artificial rearing medium to reduce costs for mass rearing the parasitoid have been ongoing [15]. Once an artificial medium has been developed, a method for harvesting the eggs will be required for use in mass rearing of the parasitoid. Tilden and Ferkovich [14] reported the presence of an ovipositional stimulating kairomone in the hemolymph of *H. zea*. Hemolymph incorporated into an artificial ovipositional substrate made from agarose gel drops enables a large number of ovipositional bioassays to be obtained on a routine basis. We report here progress in the isolation and the identification of the OSK* and the results of our studies assessing the viability of eggs collected using the AOS.

MATERIALS AND METHODS

Insect Rearing, Hemolymph Collection, and Preparation of AOS

The host species, *H. zea*, was mass-reared on a pinto-bean-based diet in our laboratory according to previously described procedures [22]. In initial experiments, *M. croceipes* was reared in the laboratory as described by Ferkovich and Dillard [19]. Due to the large numbers of parasitoids required for the bioassays, additional parasitoids were purchased from the Delta States Research Center, USDA, ARS (Stoneville, MS). The pupae of the parasitoids were held for emergence in an environmental chamber maintained at 26°C ± 1°C, 70% RH, and a photoperiod of 15:9 (L:D). Males and females were caged together and were provided with water and honey.

Hemolymph was collected from fourth and fifth instar *H. zea* larvae by cutting a proleg and drawing the hemolymph into a capillary tube. To prevent melanization, 10 μl of a 0.05 mg/ml solution of PTU in methanol was added per 500 μl of hemolymph held on ice. Samples were centrifuged at 13,000*g* in a Fisher (Fisher, Pittsburgh, PA) microcentrifuge for 10 min to remove cellular material and were stored at −20°C. Hemolymph used in egg viability studies was obtained from fifth instar larvae that were held for 5 min in a water bath at 63°C. This treatment prevented melanization of the hemolymph when it

*Abbreviations used: AOS = artificial oviposition substrate; HE = hemolymph equivalents; HPLC = high performance liquid chromatography; K_d = distribution coefficient; kDa = kilodaltons; Me_2Cl_2 = methylene chloride; OSK = ovipositional stimulating kairomone; PTU = 1-phenyl-2-thiourea.

was incubated with the parasitoid eggs in vitro and was an alternative to using PTU, which was toxic to the eggs.

AOS's were prepared as described earlier [14], with certain modifications. Agarose (Sigma, St. Louis, MO) was dissolved in boiling water to produce a 1% gel solution. Drops (30 μl) of the molten agar were dispensed onto the bottom of a plastic petri dish using a positive displacement pipette. Before the agar drop cooled, a small bubble (ca. 15 μl) of air was made in the top of the AOS drop with the pipette. After hardening, the bubble was broken, and the test material (10 μl) was applied to the concave indentation and allowed to diffuse throughout the drop for 45–60 min before female wasps were introduced into the dish.

For tests of the egg viability, AOS's were prepared from a gel solution prepared with Goodwin's isotonic IPL-52B tissue culture media (Hazleton Research Products, Lexena, Kansas) [23] in place of water. Ten microliters of heat-treated and filter-sterilized hemolymph [15] were applied to the well of the AOS.

Bioassay

Each replication consisted of one female wasp (3–5 days postadult emergence and taken from cages containing both sexes) in a 9 cm diameter plastic petri dish containing one AOS. Typically, six treatments with five replications per treatment (30 petri dishes) were utilized. Petri dishes were placed in a plastic shoe box with wet paper toweling on the bottom to prevent dehydration of the agar drops. The shoe (boxes) dishes were held in an environmental chamber at 26–27°C. Testing began at 2–4 h after the onset of photophase. The number of eggs laid was recorded after 2 and 4 h. The percentage of females ovipositing (at least once) was determined from the number of eggs laid in each single AOS per petri dish.

Serial dilutions of *H. zea* HE were made with deionized water to establish an OSK dose-response curve. Ten microliters of one of the following five dilutions were applied to the AOS: 1 μl, 0.5 μl, 0.25 μl, 0.125 μl, and 0.0625 μl HE/μl.

The effect of an extract of host feces and the tyrosinase inhibitor PTU on ovipositional activity was also examined. The six treatments were the following: deionized water, PTU in deionized water (10 μl of a 0.05 mg/ml solution in methanol added to 500 μl water), feces extract (10 μl of a 0.1 mg/μl of *H. zea* feces in hexane), water with PTU + feces extract, hemolymph + PTU, and hemolymph + PTU + feces extract.

Analytical Procedures

Apolar and polar solvent distribution (K_D) studies. Hemolymph (500 μl) was extracted three times with 1.5 ml of either hexane, ethyl ether, or methylene chloride. Between each extraction, the samples were centrifuged at 3,000*g* for 5 min to remove emulsions and precipitates. The organic solvent layers were pipetted off, combined, and concentrated to 500 μl in a rotary evaporator having a bath temperature of 90°C. Material not dissolved in the organic solvent was further extracted with water. The organic, aqueous, and recombined organic/aqueous soluble material were bioassayed using the previously described protocol. In a similar manner hemolymph (500 μl) was frozen in liquid nitrogen and lyophilized overnight. The powdered material was reconstituted

with 500 µl of either methanol, tetrahydrofuran, or acetonitrile. After removal of the polar organic solvent, the remaining residue was extracted with water. Bioassays were conducted with the material obtained with polar organic solvent extraction, the residue remaining after organic solvent extraction and subsequent water extraction, and the material from a combined aqueous/organic extraction.

Ultrafiltration. After extraction of lipids, the active material was further purified using a Unisep® ultrafiltration cartridge (Ultracent-10, 10 kDa, Bio-Rad, Richmond, CA). Five-hundred microliter equivalents of the aqueous phase of solvent-extracted hemolymph were pipetted into the sample reservoir of the filter and centrifuged at 3,000*g* for 45 min. The filtrate cup was removed and the unit was inverted and centrifuged at 500*g* for 2 min. The retentate was then brought to volume with water for bioassay. The retentate and filtrate were bioassayed as previously described.

Solid phase extraction. Solid-phase extraction columns (J.T. Baker spe® columns, J.T. Baker, Phillipsburg, NJ) were examined for their capacity to purify the OSK further prior to HPLC. The solid-phase column materials examined included phenyl, octyl, cyano, amino, diol, two weak anion (amino 1°/2°) and cation (aromatic sulfonic acid) columna. The method used for the evaluation of the solid-phase material consisted of applying 200 µl of the aqueous phase of ether-extracted ultrafiltered hemolymph to the columns that had been equilibrated as follows: phenyl and octyl columns were eluted with 5 ml of methanol followed by 5 ml of HPLC grade water. OSK material was eluted from the cartridge with 10 ml of HPLC grade water. In the case of the normal-phase adsorbent columns (octyl, amino, diol), each column was conditioned with 5 ml of hexane and 5 ml of methanol prior to use. Material was eluted from the column with 5 ml of HPLC water. Cation and anion exchange cartridges were conditioned with 5 ml hexane, 5 ml methanol, 5 ml water prior to use. OSK material was eluted from the cation column with 5 ml water followed by 5 ml 0.1 N HCl, and water was used to elute material from the anion exchange column.

Column chromatography. Analytical HPLC was performed using a Kratos Spectraflow® 400 pump (Kratos, Ramsey, NJ), a Waters® refractive index detector (Model 402, Waters, Milford, MA), and a Rheodyne® injector (Rheodyne, Cotati, CA). Gel permeation chromatography was carried out using a 50 cm long by 1 cm i.d. low pressure column packed with Bio-gel® P-2 (Bio-Rad, Richmond, CA) exclusion limit 1800. Column calibration was obtained using a series of polyethylene glycols having molecular weights of 200, 560, 980, 1580, and 1800. One-thousand microliters of hemolymph equivalent (1,000 µl HE) of the aqueous phase of ether extracted and ultrafiltered hemolymph were applied to the column and the column was then eluted with HPLC water at 0.20 ml/min. Ten 2.5 ml fractions were collected, lyophilized, and bioassayed at 10 µl HE. Additional HPLC was performed using a carbohydrate analysis column (Aminex® HPX-87C, 300 × 7.8 mm i.d., Bio-Rad, Richmond, CA) maintained at 85°C. The column was eluted with a 10 mM calcium sulfate buffer, pH 5.35, at a flow rate of 0.6 ml/min. HPLC also was done using a phenyl column (250 × 5.8 mm i.d., J.T. Baker, Phillipsburg, NJ). The phenyl column was eluted with a gradient consisting of 15 ml of methanol followed by a linear increase of

water to a final 1:1 ratio of methanol-water. Ten 5 ml fractions were collected from each column, lyophilized and bioassayed at 10 µl HE.

Tests of egg viability. Eggs were teased out of AOS's prepared with Goodwin's tissue culture medium and tested for viability as described below. After removal from the AOS's, the eggs were rinsed five times in 20 µl drops of Goodwin's media, then transferred to 0.1 ml of medium that had been precultured for 24 h with fat body explanted from surface-sterilized, nonparasitized, prewandering fifth instar *H. zea* larvae following the procedures of Greany [15]. The eggs were examined daily for progress in their development and hatchability.

RESULTS

Bioassay

The graph of percent oviposition into an AOS versus increasing amount of hemolymph equivalents on the AOS during a 4-h time period is shown in Figure 1. Regression analysis of this data resulted in a equation $Y = 31.2\ Ln\ X + 11.2$ with correlation coefficient of $r^2 = 0.94$. Females did not lay eggs in treatments containing only water, PTU in water, feces extract, or water + PTU + feces extract (Table 1). Addition of the feces extract to host hemolymph did not affect the percent oviposition nor the number of eggs oviposited compared to PTU + hemolymph (Table 1).

Sample Concentration and Initial Purification

The results from the rotary evaporation and lyophilization experiments indicated there was no apparent loss in activity of the material using either method of concentration and reconstituting the material with water (Table 2). No appar-

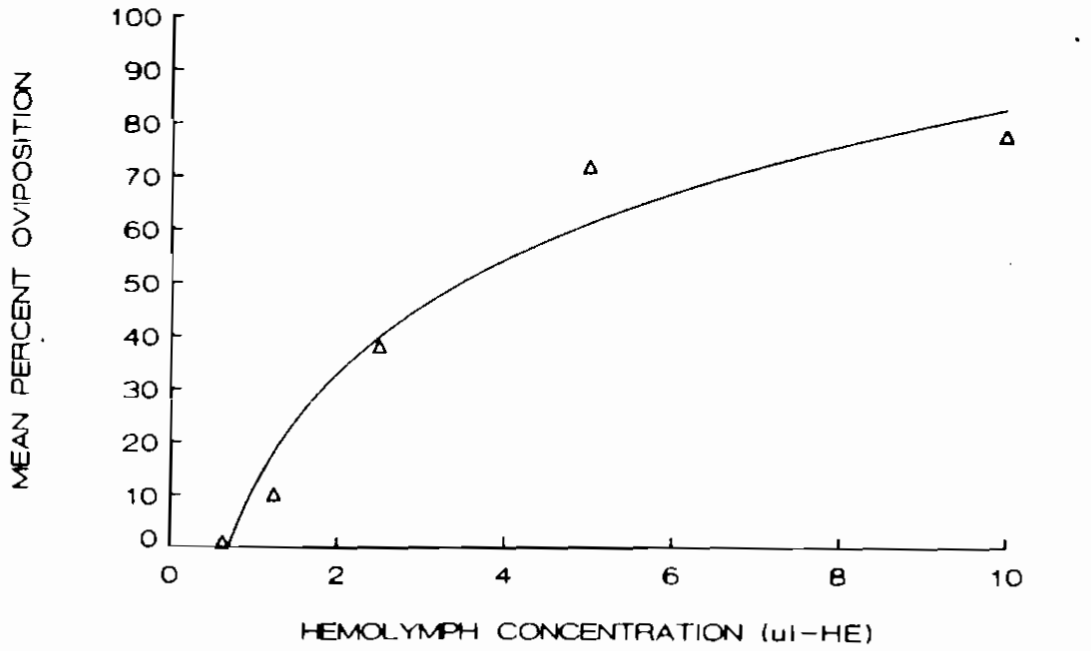

Fig. 1. Percentage of *M. croceipes* ovipositing in artificial substrate with increasing amount of *H. zea* hemolymph. Regression analysis was based on the mean of 30 replicates ($Y = 31.1\ ln\ X + 11.2$; $r^2 = 0.94$).

TABLE 1. Oviposition Activity of *M. croceipes* to *H. zea* Hemolymph With and Without *H. zea* Feces and Various Control Substances

Treatment[a]	Mean ± SD	
	Eggs/ovipositing ♀	% oviposition
Water	0.0	0.0
PTU[b]	0.0	0.0
Feces hexane extract	0.0	0.0
PTU + feces extract	0.0	0.0
PTU + feces + hemolymph	8.1 ± 3.0	53.3 ± 14.3
PTU + hemolymph	6.7 ± 2.3	56.7 ± 22.1

[a]Artificial oviposition substrate containing 10 µl of treatment. Six replicates each treatment. Replicate consists of five bioassays.
[b]PTU = phenylthiourea.

ent decrease in activity was noted when the material was heated to 90°C for rotary evaporation of solvents.

Extraction of hemolymph with hexane or ether resulted in similar OSK activity when compared with the crude hemolymph (Table 2). Active material which remained in the aqueous phase was less viscous when ether extraction was used as the initial purification step. Results obtained when methylene chloride was used to extract crude hemolymph gave varying results. Only a small amount of activity remained in the aqueous phase (hemolymph—extracted with Me_2Cl_2) after extraction with this solvent, and no activity was found in the organic layer (Me_2Cl_2). Recombination of both layers (aqueous and organic) increased the activity over that found in the aqueous layer but was reduced when compared to either the crude hemolymph, hemolymph extracted with hexane, or ether (Table 2). These results indicated that methylene chloride

TABLE 2. Oviposition Activity of *M. croceipes* to Various Purifications of *H. zea* Hemolymph

Treatment[a]	n[b]	Mean ± SD	
		Eggs/ovipositing ♀	% oviposition
Hemolymph	10	6.3 ± 5.0	60.0 ± 31.4
Hemolymph rotoevaporated	6	5.8 ± 5.8	60.0 ± 16.2
Hemolymph lyophilized	7	6.6 ± 5.6	54.6 ± 39.4
Hemolymph	22	5.4 ± 3.6	55.5 ± 24.8
Hemolymph—hexane extracted	6	4.4 ± 1.7	63.3 ± 13.7
Hemolymph—ether extracted[c]	16	6.0 ± 5.2	48.8 ± 29.3
Hemolymph + ether extract[d]	8	5.2 ± 2.5	46.5 ± 32.3
Ether extract of hemolymph	4	0.0	0.0
Hemolymph - Me_2Cl_2 extracted	10	1.6 ± 1.2	14.0 ± 15.6
Hemolymph + Me_2Cl_2 extract	7	4.7 ± 2.7	28.6 ± 18.1
Me_2Cl_2 extract of hemolymph	7	0.0	0.0
Molecular weight filtration >10K	6	0.0	0.0
Molecular weight filtration <10K	6	2.7 ± 1.4	53.3 ± 22.1

[a]Artificial oviposition substrate containing 10 µl of hemolymph, extracted hemolymph, or solvent used in extraction.
[b]No. (n) of replicates. Each replicate consists of five bioassays.
[c]Hemolymph which has been extracted with ether.
[d]Hemolymph extracted with ether combined with the ether-extracted material.

extraction was not suitable for initial extraction because of the overall decrease in OSK activity. This extraction method was not used during the remainder of this investigation.

Efforts to partition the concentrated, active material into polar organic solvents such as methanol, tetrahydrofuran, or acetonitrile were unsuccessful. Furthermore, water extraction of the material remaining after polar organic solvent extraction with water had little or no activity by itself or when combined with the organic solvent.

Ultrafiltration of the aqueous phase of the ether extracted hemolymph using a 10 kDa cutoff membrane indicated that the kairomone activity was in the filtrate the molecular weight therefore was less than 10 kDa (Table 2). Further purification of this material was obtained using a gel permeation column with an exclusion limit of 1.8 kDa. All activity was recovered in a fraction with a calculated molecular weight range of less than 300 (Fig. 2).

Solid-Phase Purification and HPLC

Of the eight different solid-phase adsorbents tested for their potential use for HPLC purification of the OSK, the normal-phase diol adsorbent column eluted with water resulted in material having the highest percent oviposition (Fig. 3) The second-best material found for purifying the OSK was the reverse-phase phenyl adsorbent (Fig. 3). Both of these adsorbents produced fractions with a higher activity than crude hemolymph (Fig. 3). Material eluted with water from the normal-phase cyano adsorbent had activity equal to the hemolymph control, followed by the octyl reverse-phase adsorbent (Fig. 3). Use of the amino normal-phase adsorbent resulted in a significant loss of OSK activity (Fig. 3). Purification of the OSK using ion-exchange adsorbents resulted in

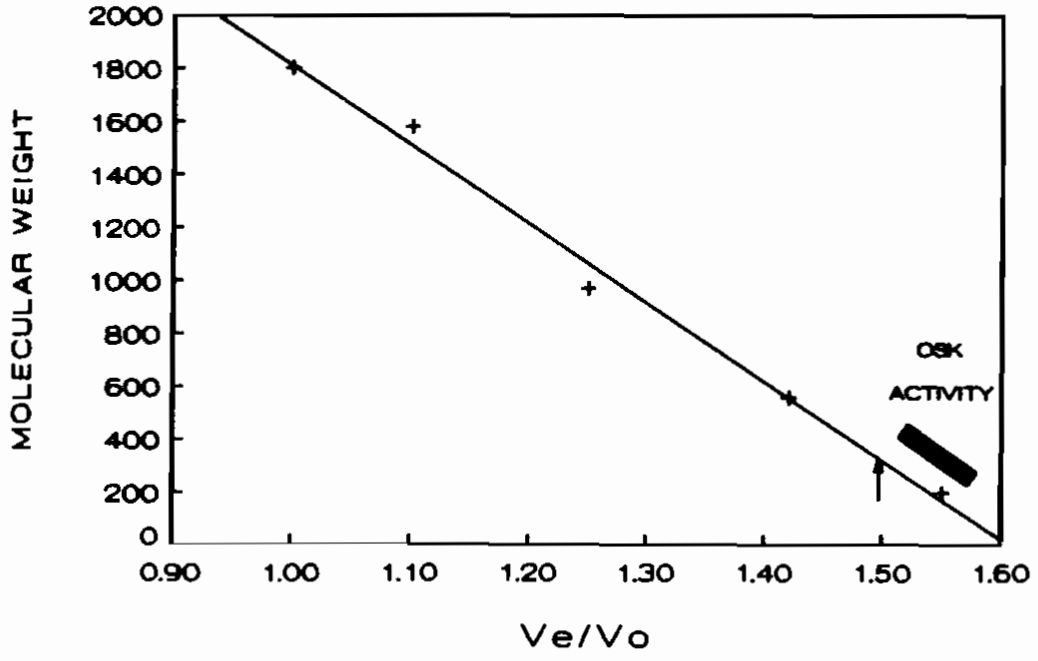

Fig. 2. Plot of standards used to qualify molecular weight range of fractions eluted from the gel permeation column. Line is the result of a linear curve fit ($Y = -2{,}973.4*X + 4{,}780.5$; $r^2 = 0.98$) Dark bar indicates the fractions having OSK activity and the arrow indicates the elution of trehalose, which has a molecular weight of 342 (Ve/Vo = 1.49).

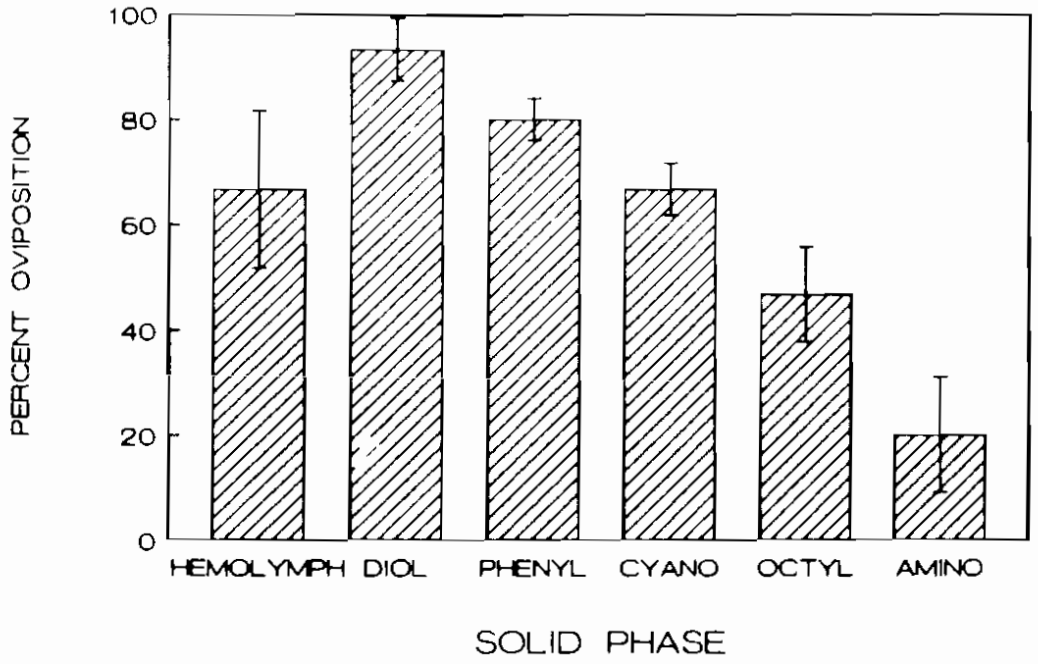

Fig. 3. Percentage of *M. croceipes* ovipositing in artificial substrate containing 10 µl-HE of material eluted from five different solid-phase adsorbents. Number of replicates equals six. Error bars indicated the standard deviation.

less than a 10% recovery of activity using the solvent system described and is under further investigation.

Further fractionation of the OSK was investigated using two types of HPLC columns. Active material from hemolymph that had been ether-extracted, centrifuged through the 10 kDa membrane, eluted from phenyl solid-phase cartridge with water and collected from a gel permeation column was chromatographed on a carbohydrate column (Aminex® HPX-87C) and a reverse-phase phenyl column. Activity was lost when the active material was chromatographed on the carbohydrate column regardless of the eluent used. Elution of active material from the phenyl column was obtained using a methanol-water gradient. Results from this separation indicated that the OSK was composed of at least two components (Table 3). Activity equivalent to the crude hemolymph was found when all fractions 1 through 25 were combined (3–50 ml). Similarly, complete activity was obtained when three fractions were combined (Table 3).

TABLE 3. Oviposition Activity of *M. croceipes* to Fractions From Host Hemolymph Purified by HPLC on a Phenyl Column

		Mean ± SD	
	n[a]	Eggs/ovipositing ♀	% oviposition
Hemolymph	8	3.7 ± 2.1	60.3 ± 23.7
Fraction 1-25 (3-50 ml)	8	5.5 ± 2.3	53.3 ± 9.3
Fraction 2 (5-7 ml)	8	1.3 ± 1.2	30.0 ± 19.1
Fractions 9,10 (19-22 ml)	8	0.2 ± 0.1	13.3 ± 9.4
Fractions 2,9,10 (5-7 & 19-22 ml)	8	6.4 ± 3.1	56.7 ± 13.7

[a]No. (n) of replicates. Each replicate consists of five bioassays.

No peaks were seen in the chromatograms obtained using a refractive index detector for the material being eluted from the column.

Egg Viability

Overall, 37% of the eggs laid in AOS's hatched and a similar percentage hatched of the total number of eggs derived from the host tested hatched (Table 4).

DISCUSSION

Tilden and Ferkovich [14] previously demonstrated that hemolymph from *H. zea* incorporated into an artificial substrate such as agar resulted in oviposition by *M. croceipes* females. This investigation supports and provides further definition of the nature of the ovipositional kairomone. In addition, hexane extracts of host feces were found to have no effect on the activity of the OSK in the bioassays although feces have been shown to contain an important factor(s) for the host selection process [13,20,24–26]. Apparently, host feces is not required in eliciting egg laying in *M. croceipes*.

We have developed a general analytical protocol for isolation of the OSK that consists of an initial extraction of organic soluble materials using ethyl ether. Attempts to characterize and purify the OSK further based on the solvent distribution coefficient (K_d) of the active material in polar organic solvents suggested that at least one of the chemicals involved in OSK activity is labile in the presence of polar organic solvents. Conversely, extraction with apolar solvents such as ether or hexane did not reduce OSK; consequently, ether was used in the initial purification step in conjunction with ultrafiltration. The active material remained in the aqueous phase. This material was less viscous, suggesting that a significant amount of material not related to OSK activity (lipid and hydrocarbon-like materials) were effectively removed at this extraction step. Fractionation of the hemolymph sample by gel permeation after these steps revealed an apparent molecular weight of 100–300 daltons. Several solid-phase adsorbent cartridges were found to be useful as a preliminary step in determining what analytical column to use in further purification and isolation of the kairomone. The normal-phase diol and the reverse-phase phenyl adsorbents appear to have the highest potential for purification of the OSK. HPLC with a reverse-phase phenyl column indicated that the OSK may be composed of at least two components because oviposition activity was obtained only when the fractions having different retention volumes were com-

TABLE 4. Developmental Competence of AOS vs. Host-Derived Eggs

Source of eggs	No. of replications	No. of eggs/replication	% Hatch avg. SD
AOS*	7	5	37.6 ± 14.7
Host**	3	5	40.0 ± 20.0

*Fat body (18.3 mg avg.) explanted from surface-sterilized, nonparasitized prewandering fifth instar *Heliothis zea* larvae was added to 0.1 ml Goodwin's IPL-52B medium 24 h prior to addition of AOS eggs. Fat body was kept with eggs throughout the test.

**Eggs were dissected from prewandering fifth instar host larvae immediately (< h) after parasitization and tested for viability similar to AOS eggs.

bined. Interestingly, the carbohydrate column provided good recovery and resolution of many sugars and neutral and acidic amino acids (unpublished); however, the active material was lost when chromatographed on this column. Further purification of the OSK using several other HPLC columns is in progress to provide materials suitable for identification of the ovipositional kairomone of *M. croceipes*.

Our results showed that the AOS's formed from agar and IPL-52B tissue culture medium permit the collection of viable eggs from an endoparasitoid under artificial conditions. This advance not only saves time by avoiding the tedium of dissecting host larvae to collect eggs for experimental purposes, but also presages its use in mass rearing. Further development of the technique will be required for improving the 37% rate of hatch of the AOS-derived eggs for use in conjunction with purified OSK in a mass-rearing program.

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